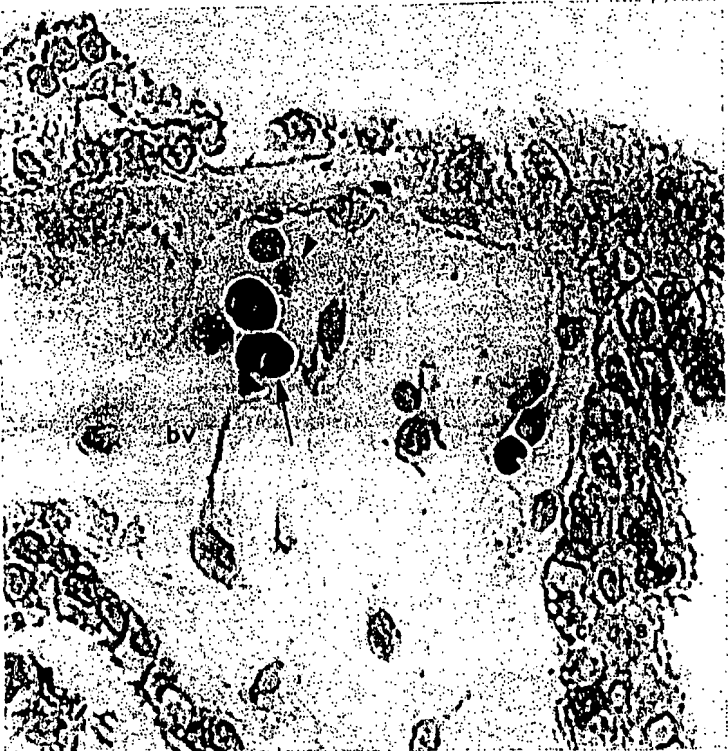


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(57) Abstract <p>A method of identifying embryonic or fetal red blood cells in a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising determining which cell or cells contain or express an adult liver component. A method of isolating embryonic or fetal red blood cells from a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising isolating the cells which contain or express an adult liver component. A method of determining a fetal abnormality the method comprising identifying or isolating embryonic or fetal cells according to the above methods and analysing said embryonic or early fetal cells for said abnormality. Use of a means for determining whether a cell contains or expresses an adult liver component for identifying or isolating an embryonic or fetal red blood cell.</p>			
			

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PRENATAL DIAGNOSTIC METHODS

The present invention relates to diagnostic methods, in particular to methods of prenatal diagnosis and to reagents for use in such methods.

5

Prenatal diagnosis is carried out widely in hospitals throughout the world. Existing procedures such as fetal, hepatic or chorionic biopsy for diagnosis of chromosomal disorders including Down's syndrome, as well as single gene defects including cystic fibrosis are very invasive and carry a not
10 inconsiderable risk to the foetus and a small risk to the mother.

Amniocentesis, for example, involves a needle being inserted into the womb to collect cells from the embryonic tissue or fluid. The test, which can detect Down's syndrome, carries a miscarriage risk estimated at 1 %.

15

Fetal therapy is in its very early stages and the possibility of very early tests for a wide range of disorders would undoubtedly greatly increase the pace of research in this area. Current fetal surgical techniques have improved, making fetal surgery for some genetic problems like spina
20 bifida and cleft palate very feasible. In addition, relatively simple effective fetal treatment is currently available for other disorders for example 21-hydroxylase (treatment with dexamethasone) and holocarboxylase synthetase (treatment with biotin) deficiencies, as long as detection can take place early enough.

25

Thus, relatively non-invasive methods of prenatal diagnosis are an attractive alternative to the very invasive existing procedures. A method based on maternal venepuncture should make earlier diagnosis more widely available in the first trimester, increasing options to parents and
30 obstetricians (because genetic disorders could be detected earlier and more

safely), and allowing the eventual development of specific fetal therapy.

The possibility of recovering fetal cells from the maternal circulation has excited general interest as a possible means, non-invasive to the fetus, of
5 diagnosing fetal anomalies (Simpson & Elias (1993) *J. Am. Med. Assoc.* 270, 2357-2361). Initial interest was directed towards trophoblastic detection systems but separation of those cells by flow cytometry has been unreliable as maternal lymphocytes appear to absorb proteins released by trophoblastic cells (Mueller *et al* (1990) *Lancet* 336, 197-200; Covone *et al*
10 *al* (1984) *Lancet* 13 October edition, 841-843). More recently, attention has focused on the development of methods to isolate fetal blood cells for cytogenetic analysis particularly nucleated fetal erythrocytes as their numbers exceed those of fetal lymphocytes in the maternal circulation. Identification of fetal red blood cells in maternal blood has been described
15 ie in a male fetus with Y centromere probes to identify fetal cells or amplification of Y-specific DNA sequences (Price *et al* (1991) *Am. J. Obstet. Gynecol.* 165, 1731-1735; Zheng *et al* (1993) *J. Med. Genet.* 30, 1051-1056; Hamada *et al* (1993) *Hum. Genet.* 91, 427-432; Cheung *et al* (1996) *Nature Genetics* 14, 264-268; and Williamson (1996) *Nature*
20 *Genetics* 14, 239-249) or karyotype identification in trisomic conditions (for example, see Bianchi *et al* (1992) *Hum. Genet.* 90, 368-370).

Hume *et al* (1995) *Early Human Development* 42, 85-95 shows that the microsomal glucose-6-phosphatase enzyme protein is present in human
25 embryonic and fetal red blood cells.

Pazouki *et al* (1996) *Acta histochem. (Jena)* 98, 29-37 attempts to identify fetal nucleated red blood cells using combined immunocytochemistry using a human fetal haemoglobin antibody and an *in situ* hybridisation method
30 using X and Y chromosome probes.

Hume *et al* (1996) *Blood* 87, 762-770 describes a study of the expression of endoplasmic reticulum proteins in human embryonic and fetal red blood precursors.

5 Wachtel *et al* (1991) *Human Reproduction* 6, 1466-1469 describes the use of PCR to identify Y-specific DNA sequences in maternal cells isolated by cell sorting with transferrin receptor antibody and glycophorin A antibody.

10 Yeoh *et al* (1991) *Prenatal Diagnosis* 11, 117-123 describes the detection of fetal cells in the maternal circulation by enzymatic amplification of a single copy gene that was fetal specific.

15 Holzgreve *et al* (1992) *J. Reprod. Med.* 37, 410-418 shows that the transferrin receptor antigen alone is not sufficient for enrichment of fetal nucleated erythrocytes and points out that the reproducibility and reliability of the techniques are still limited, mainly due to the lack of very specific cell markers.

20 Zheng *et al* (1993) *J. Med. Genet.* 30, 1051-1056 describes the use of a magnetic activated cell sorter (MACS) to enrich fetal nucleated erythrocytes using mouse monoclonal antibodies specific for CD45 and CD32 to deplete leucocytes from maternal blood. The paper points out that significant maternal contamination was present even after MACS
25 enrichment preventing the accurate analysis of fetal cells by interphase fluorescence *in situ* hybridisation (FISH).

Tomoda (1964) *Nature* 202, 910-911 describes the demonstration of fetal erythrocytes by immunofluorescent staining.

There exists a need for improved methods for identifying fetal cells in maternal blood in order to carry out prenatal diagnosis.

We attempted to isolate embryonic and fetal nucleated red blood cell from
5 maternal blood with the established immuno-magnetic sorting using
sequentially anti-CD45 and anti-CD18 antibodies to remove white blood
cells and then anti-CD71 (transferrin receptor) antibodies to enrich fetal
nucleated red blood cells. We were very disappointed to discover that
immuno-magnetic sorting, with the anti-CD71 antibody, did not purify
10 embryonic red blood cells of the megaloblastic series. The advantage of
purifying megaloblastic cells is that they are the predominant red blood
cell type in the embryo and early fetus and that they are nucleated whereas
the vast majority of adult red blood cells are normocytic and non-
nucleated.

15
In subsequent conventional immunohistochemistry, we found that CD71
interactions with megaloblasts were very weak presumably explaining the
poor purification of embryonic cells with this antibody. This is a very
major problem in the current use of maternal blood for early diagnosis.
20 We have shown that the nucleated megaloblastic series predominates in
early development compared to nucleated normoblasts. This means that
using the conventional antibodies ie anti-CD71, it is very difficult to
obtain pure nucleated haemopoietic cells arising from the early conceptus.
Anti-CD71 does not immunoreact with the majority of these early cells
25 making this technique possible in specialised research labs with specialist
staff and equipment, but not practical in routine service laboratories.

The use of anti-CD71 is described in Cheung *et al* (1996) *Nature Genetics*
14, 264-268 and is reviewed by Williamson (1996) *Nature Genetics* 14,
30 239-240. Having to look at thousands of cells to find a few fetal ones on

slides is a problem with this approach, as is the fact that anti-CD71 antibodies are not selective enough for fetal cells and do not react with embryonic cells.

- 5 An object of the present invention is the provision of improved methods for identifying fetal cells in, and isolating them from, maternal blood. In particular, an object of the invention is the provision of methods of identifying and isolating embryonic or early fetal red blood cells, and analysing the cells for fetal abnormalities.

10

A first aspect of the invention provides a method of identifying embryonic or fetal red blood cells in a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising determining which cell or cells contain or express an adult liver
15 component.

A second aspect of the invention provides a method of isolating embryonic or fetal red blood cells from a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising
20 isolating the cells which contain or express an adult liver component.

It has not previously been proposed that embryonic or early fetal nucleated red blood cells are functioning as adult liver cells while circulating in the bloodstream.

25

Suitably, the sample containing maternal blood cells and embryonic or fetal red blood cells or both is a sample of blood from a pregnant female.

Preferably the fetal red blood cell is an early fetal red blood cell.

30

The pregnant female may be any mammal and in particular a mammal of commercial or agricultural importance or a domesticated mammal. Suitably, the mammal is a horse, cow, sheep, pig, goat, dog, cat or the like. The basic pattern of haematological development in the embryo and
5 fetus is the same for all mammals.

Preferably the pregnant female is human.

A particular advantage of the present invention is that the methods
10 identify, and can be used to isolate, embryonic and early fetal red blood cells at an early stage of gestation. Thus, it is preferred if the maternal blood sample is taken from the pregnant female at an early stage of pregnancy. In the case of a pregnant human female it is preferred if the sample is taken in the first trimester.

15

In general the earlier in pregnancy, whether for potential fetal therapy or the option of termination, the better (ideally less than 10 weeks gestation). A further practical reason is that the means of termination of pregnancy is technically easier at earlier gestations and with less physical and
20 psychological side-effects. There is no upper limit for intrauterine diagnosis of fetal anomalies and even late in pregnancy treatment may still be beneficial *in utero* or the immediate newborn period. The ontogeny of nucleated embryonic and fetal cells clearly indicates that the percentage of those cells in the embryo/fetus is higher in the first trimester than later in
25 pregnancy. However, the total fetal blood volume increases with gestation and proportionally fetal to maternal transfusion volumes may be greater.

The detailed structure of the developing human conceptus, sufficient for accurate dating in days, has only been described in detail up to 56 day
30 post-ovulatory days and the descriptive term, embryo, will be used as the

convention for this developmental staging procedure (O'Rahilly & Muller (1987) *Developmental Stages in Human Embryos*, Publication 637, Washington: Carnegie Institute of Washington). The descriptive term, fetus, will be used for the remainder of human intrauterine development
5 to term (>37 completed weeks gestation), with developmental age (to the nearest week) an estimate based on size, including crown-heel, crown-rump and heel-toe measurements and *Growth of the External Dimensions of the Human Body in the Fetal Period*, Minneapolis: University of Minnesota Press) menstrual history and ultrasound dating of pregnancy.

10

The methods of the invention are particularly suited to identify, and can be used to isolate, embryonic and fetal red blood cells of the nucleated megaloblastic series which predominate in early development compared to nucleated normoblasts. However, nucleated normoblasts may also be
15 isolated or identified by the method with equal advantage. The proportion of megaloblasts are higher at earlier stages of pregnancy. The morphology of embryonic/early fetal megaloblast differs fundamentally from embryonic/fetal normoblasts and the small number of maternal normoblasts which could be present in the circulation. Maternal
20 megaloblasts and megalocytes are extremely rare but do occur in vitamin B12 and a folate deficiency. For these reasons embryonic/early fetal megaloblasts are preferred but embryonic/early fetal normoblasts would also be capable of being used.

25 The sample containing maternal blood cells and embryonic or fetal red blood cells or both may be a sample from which has been removed certain maternal blood cells. For example, adult white blood cells can be removed from maternal blood using sequentially anti-CD45 and anti-CD18 antibodies although with a relatively poor efficiency. Enrichment of
30 samples may be achieved by density centrifugation (eg Ficoll gradients)

but will not separate adult and fetal nucleated cells.

The sample may be a sample which has been enriched for fetal cells. For example, it could be a sample enriched by the use of an anti-transferrin
5 receptor antibody as described in Cheung *et al* (1996) *Nature Genetics* **14**, 264-268.

The sample (particularly when it is one which fetal or embryonic cells are to be identified in rather than isolated from) may be one which has been
10 treated in order to undertake haematological, biochemical, histochemical or molecular biological analysis or the like. For example, the sample may be a blood sample or a sample of a fraction of blood (such as one that has been enriched for fetal cells/depleted of maternal cells) which has been prepared for immunocytochemical analysis or for fluorescence *in situ*
15 hybridisation (FISH) analysis or merely has been spread on a microscope slide.

The sample containing maternal blood cells and embryonic or fetal red blood cells or both may be any suitable sample (including a fluid) which
20 contains such cells. For example, the sample may be urine from a patient with haematuria, amniotic fluid or fetal blood.

By "adult liver component" we mean a component of an adult liver cell which is predominantly associated with the adult liver and, if it is found
25 at all in other tissues of the adult, it is either found at low levels in that other tissue compared to the liver or that the mass of the other tissue in which the said component is found, compared to the mass of the liver, is low so that the total amount of the adult liver component is higher in the whole liver compared to the total amount in the whole other tissue. When
30 the adult liver component is found at low levels in that other tissue it is at

least 10-fold higher in the liver, preferably at least 100-fold higher in the liver, compared to that other tissue. When the adult liver component is found at similar levels to the liver in other tissues, that other tissue has 1/10 of the mass of the liver, more preferably 1/25 of the mass of the liver.

While the kidney has many functions not related to liver, it can also carry out, to a lesser extent, some liver functions such as gluconeogenesis. It is particularly preferred if, in the above definition of adult liver component, the "other tissue" is not kidney.

For example, glucose-6-phosphatase is an adult liver component. Glucose-6-phosphatase levels in liver are much higher than glucose-6-phosphatase levels in total pancreas. However, glucose-6-phosphatase levels in islet cells (which are only a small proportion of cells in pancreas) can be as high as in liver. Similarly, GLUT2 is an adult liver component which is expressed in islet cells. Both are considered to be predominantly liver proteins in the sense that the total mass of islets in the human body is minute compared to the total mass of liver. The level of adult liver component is measured per unit cell fraction or per unit cell or per unit tissue.

The adult liver component is, therefore, typically, adult liver selective or adult liver specific.

The adult liver component may be any suitable such component and may include protein, RNA, carbohydrate entities and metabolites provided that these are predominantly associated with the adult liver and, if it is found at all in other tissues of the adult, it is either found at low levels in that other tissue compared to the liver or that the mass of the other tissue in

which the component is found compared to the mass of the liver is low so that the total amount of the adult liver component is higher in the whole liver compared to the total amount in the whole other tissue.

- 5 The embryonic or fetal cells may be identified or isolated according to the methods of the invention by the detection of, or binding to, of one or more adult liver components as defined.

10 It is particularly preferred if the adult liver component is substantially absent from maternal cells of the maternal blood. Preferably, compared to embryonic or fetal red blood cells, maternal cells of the maternal blood contain less than 1% of the adult liver component on a per cell basis; more preferably they contain less than 0.1% on a per cell basis.

- 15 It is possible, when the liver has been acutely damaged by trauma, cells or clumps could be disseminated in the bloodstream. Thus, it is preferred if the sample is not a sample of blood from a female whose liver has been damaged so as to release liver cells into the blood. It is also preferred that the sample is not any other sample that contains adult liver cells.

20

Liver damage is assessed by plasma estimation of routine liver function tests, eg alanine aminotransferase activity. Morphologically, the appearance of a circulating adult liver cell and an embryonic nucleated red blood cell are sufficiently distinct to allow discrimination.

25

Preferably, the adult liver component is a protein.

Preferably, the adult liver protein is any one of a microsomal glucose-6-phosphatase enzyme, another protein component of the glucose-6-phosphatase system including the phosphate or glucose or glucose-6-

30

phosphate transporters, a uridine diphosphate-glucuronosyltransferase (UDPGT), a cytochrome P450 isozyme (P450), nicotinamide adenine dinucleotide phosphate (NADP) cytochrome P450 reductase (P450 reductase), glucose transporter 2 (GLUT2), a P-glycoprotein, a MDRP
5 (multidrug resistance protein), a MRP (multidrug resistance-like protein), γ -glutamyl transpeptidase, a lipoprotein receptor, an alkaline phosphatase, a bile salt transporter, a bile acid transporter, a hormone receptor, a multiple organic ion transporter (MOAT; equivalent to MRP), a bilirubin transporter (bilitranslocase) or bilirubin conjugate (eg bilirubin
10 glucuronide) transporter (equivalent to MRP).

The liver plasma membrane contains transporters for a wide variety of drugs, xenobiotics and endogenous compounds because they are taken up by the liver which is their major site of metabolism. Similarly, the
15 conjugated metabolites are then exported back out across the liver plasma membrane. The transporters, many of which are adult liver components as defined, are suitable targets for the practice of the methods of the invention. It will be appreciated that further transporters of this type will be purified and their corresponding cDNAs and genes cloned. The
20 invention contemplates that the further, as yet unknown transporters, will be suitable targets.

In the case where there are isozymes of a particular protein or class of proteins which are not adult liver selective or adult liver specific, it is the
25 adult liver selective or adult liver specific isozymes which are used. For example, certain P450s are not adult liver selective or adult liver specific and so, in the practice of the invention, it is those P450s which are adult liver selective or adult liver specific which are relevant. Typically, xenobiotic-metabolising P450s are liver-selective or liver specific.

Suitably, particularly when the said embryonic or fetal red blood cells are to be isolated from the sample, the adult liver component is a cell surface component. It will, nevertheless, be appreciated that the said cell surface components are useful for both isolation and identification purposes, and
5 cell surface components are preferred for both isolation and identification purposes.

Preferably, the cell surface component is a plasma membrane protein which is predominantly associated with the adult liver plasma membrane
10 and, if it is found at all in other tissues of the adult, it is found at low levels.

Conveniently, the adult liver plasma membrane protein is any of GLUT2, a P-glycoprotein, a MDRP, a MRP, γ -glutamyl transpeptidase, a
15 lipoprotein receptor, an alkaline phosphatase, a bile salt transporter, a bile acid transporter, a hormone receptor, a MOAT, a bilirubin transporter or a bilirubin conjugate transporter, all as defined above.

If the transferrin receptor is an adult liver component as defined,
20 preferably the adult liver component is not transferrin receptor.

In order to identify or isolate the embryonic or fetal red blood cell it is preferred if said sample is contacted with a binding moiety which binding moiety binds to said adult liver component and said embryonic or fetal cell
25 is identified in, or isolated from, the sample by virtue of being bound to the binding moiety.

It will be appreciated that the embryonic or fetal red blood cells may be identified in other ways.

For example, many of the adult liver components are enzymes and so the cells can be identified by the presence of the enzyme. Suitably, histochemical stains for glucose-6-phosphatase may be used. Also suitably assays for UDP glucuronosyltransferase are useful.

5

The invention also contemplates identifying the cells by detecting adult liver components which are mRNAs by using, for example, reverse transcriptase polymerase chain reaction (RT-PCR).

- 10 In a further preferred embodiment the adult liver component is detected intracellularly. For example, when the adult liver component is an enzyme, it is convenient to use a substrate (which enters the cell which cell may or may not be permeabilised depending on the substrate) and which, when metabolised by the said enzyme, gives a fluorescent or
- 15 coloured product or a product which can readily be identified. It will be appreciated that in this embodiment the embryonic or early fetal red blood cells will be fluorescent or coloured (or marked in some other way) by virtue of the presence of the said product produced by the said enzyme. Fluorescent cells can be separated from non-fluorescent cells using a
- 20 FACS sorter. Substrates for glucose-6-phosphatase which give rise to a coloured product are known in the art.

- In addition to individual established protein components of adult liver, as potential sources of antibodies for embryonic and fetal cell isolation and/or
- 25 identification, the following approach is also useful. Human and/or other mammalian liver plasma membranes are isolated by differential centrifugation from homogenised liver (or by other techniques known in the art) and either (a) directly used to raise antibodies or (b) subfractionation of liver plasma membranes is carried out prior to raising
- 30 antibodies against particular components. Such antibodies will be

polyspecific polyclonal antibodies which bind to the adult liver plasma membrane and, according to the invention, to the plasma membrane of embryonic and fetal red blood cells.

- 5 It will be appreciated that mixtures of defined antibodies directed at adult liver plasma membrane components are also useful in the methods of the invention.

Preferably, the antibodies bind to portions of the adult liver plasma
10 membrane components which are exposed on the surface of the cell.

Conveniently, the said binding moiety is an antibody or fragment or derivative thereof.

- 15 Monoclonal antibodies which will bind to many of these antigens (whether protein antigens or non-protein antigens) are already known but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab
20 fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and*
25 *Applications*", J G R Hurrell (CRC Press, 1982).

Chimaeric antibodies are discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium Part 2*, 792-799).

- 30 Polyclonal antibodies are useful in the methods of the invention.

Monospecific polyclonal antibodies are preferred. Suitable polyclonal antibodies can be prepared using methods well known in the art.

5 Fragments of antibodies, such as Fab and Fab₂ fragments may also be used as can genetically engineered antibodies and antibody fragments.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by
10 "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

15 That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science*
20 **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques
25 involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner
30 domains are linked via a flexible oligopeptide.

Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

- 5 Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.
- 10 It will be appreciated that when the adult liver component is a receptor the receptor, and hence the embryonic or fetal red blood cell can be identified using a ligand for the receptor. For example, the cognate hormone is a ligand for a hormone receptor.
- 15 Typically, in a method of identifying the embryonic or fetal red blood cells, the binding moiety is detectably labelled or, at least, capable of detection. For example, the binding moiety is labelled with a radioactive atom or a coloured molecule or a fluorescent molecule or a molecule which can be readily detected in any other way. The binding moiety may
20 be directly labelled with a detectable label or it may be indirectly labelled. For example, the binding moiety may be an unlabelled antibody which can be detected by another antibody which is itself labelled. Alternatively, the second antibody may have bound to it biotin and binding of labelled streptavidin to the biotin is used to indirectly label the first antibody.
- 25
Typically, in a method of isolating the embryonic or fetal red blood cells, the binding moiety is immobilised on a solid support so that the embryonic or early fetal red blood cells can be isolated by affinity binding. Conveniently, the solid support comprises any suitable matrix such as
30 agarose, acrylamide, Sepharose (a trademark) and Sephadex (a

trademark). The solid support may also be a solid substrate such as a microtitre plate or the like.

Advantageously, the binding moiety is magnetically labelled (either
5 directly or indirectly) such that, when bound, the embryonic or fetal cell
can be separated from the rest of the sample upon provision of a suitable
magnetic field. Microbeads used for magnetic cell sorting are often
termed MACS colloidal super paramagnetic microbeads.

10 Fetal or embryonic cells labelled in this way may be sorted by magnetic
activated cell sorting (MACS).

Suitably, the binding moiety is labelled with a fluorescent molecule (either
directly or indirectly) and the embryonic or fetal red blood cells are
15 isolated using a fluorescence activated cell sorter (FACS).

We have, therefore, developed tools and methods which clearly identify,
and show you that you are looking at, the correct cell type (ie embryonic
or fetal red blood cells).

20

A third aspect of the invention provides a method of determining a fetal
abnormality the method comprising identifying or isolating embryonic or
fetal cells according to the method of the first or second aspects of the
invention and analysing said embryonic or fetal cell for said fetal
25 abnormality.

In one embodiment, cells are identified using a binding moiety, or
identified by virtue of the presence of a said enzyme, as described above
and the analysis of the said fetal abnormality is carried out directly on the
30 identified cells. For example, cells can be identified

immunohistochemically using a suitable binding moiety, or using a suitable enzyme-detection system, and the analysis of the fetal abnormality is carried out *in situ* on the so identified cell using techniques such as fluorescent *in situ* hybridisation (FISH) to detect chromosomal abnormalities. The polymerase chain reaction may be used *in situ*. While fluorescent detection systems work well, it is also possible to use labelled probes and enzyme-linked detection systems.

In a particularly preferred embodiment the embryonic or fetal cells are isolated according to the second aspect of the invention. The cells isolated by this procedure are substantially all embryonic or fetal cells and, in particular, there are substantially no maternal cells present.

Analysis of fetal abnormality involves analysis of the said embryonic or fetal cells according to what potential abnormality is to be investigated.

Although the method can be used according to the invention, it is preferred that a familial defect in glucose 6-phosphatase is not detected, or that disorders of liver protein expression are not diagnosed when the cells are identified by using a binding moiety which binds to an intracellular adult liver component such as glucose-6-phosphatase.

Although the method can be used according to the invention, it is also preferred that a genetic deficiency of an endoplasmic reticulum protein is not detected when the cells are identified using a binding moiety which binds to an intracellular adult liver component.

It is preferred if the fetal cell abnormality is determined by analysing the genetic material. In particular, the genomic DNA make up of the fetal cells isolated by the method of the second aspect of the invention will be

the same as the genetic DNA make up of the somatic cells of the fetus. In one preferred embodiment chromosomal abnormalities are detected. By "chromosomal abnormality" we include any gross abnormality in a chromosome or the number of chromosomes. For example, this includes
5 detecting trisomy in chromosome 21 which is indicative of Down's syndrome, trisomy 18, trisomy 13, sex chromosomal abnormalities such as Klinefelter syndrome (47, XXY), XYY or Turner's syndrome, chromosome translocations and deletions, a small proportion of Down's syndrome patients have translocation and chromosomal deletion syndromes
10 include Pradar-Willi syndrome and Angelman syndrome, both of which involve deletions of part of chromosome 15, and the detection of mutations (such as deletions, insertions, transitions, transversions and other mutations) in individual genes. Other types of chromosomal problems also exist such as Fragile X syndrome which can be detected by
15 DNA analysis. The following table indicates certain genes, mutations in which lead to particular genetic diseases.

Disease	Defective gene
Immunodeficiency	Adenosine deaminase
	Purine nucleoside phosphorylase
Hypercholesterolaemia	LDL receptor
5	Haemophilia
	Factor IX
	Factor VIII
Gaucher's disease	Glucocerebrosidase
Mucopolysaccharidosis	β -glucuronidase
Emphysema	α 1-antitrypsin
10	Cystic fibrosis
	Cystic fibrosis transmembrane regulator
Phenylketonuria	Phenylalanine hydroxylase
Hyperammonaemia	Ornithine transcarbamylase
Citrullinaemia	Arginosuccinate synthetase
15	Muscular dystrophy
	Dystrophin
Thalassaemia	β -globin
Sickle cell anaemia	β -globin
Leukocyte adhesion deficiency	CD-18

- 20 Other genetic disorders which can be detected by DNA analysis are known such as 21-hydroxylase deficiency or holocarboxylase synthetase deficiency, aspartylglucosaminuria, metachromatic leukodystrophy, Wilson's disease, steroid sulfatase deficiency, X-linked adrenoleukodystrophy, phosphorylase kinase deficiency (Type VI glycogen
- 25 storage disease) and debranching enzyme deficiency (Type III glycogen storage disease). These and other genetic diseases are mentioned in *The Metabolic and Molecular Basis of Inherited Disease*, 7th Edition, Volumes I, II and III, Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (eds), McGraw Hill, 1995. Clearly, any genetic disease where the gene

has been cloned and mutations detected can be analysed by this embodiment of the method of the invention.

Genetic assay methods include the standard techniques of restriction
5 fragment length polymorphism assays and PCR-based assays, as well as other methods described below.

The assay may involve any suitable method for identifying mutations or polymorphisms, such as: sequencing of the DNA at one or more of the
10 relevant positions; differential hybridisation of an oligonucleotide probe designed to hybridise at the relevant positions of either the wild-type or mutant sequence; denaturing gel electrophoresis following digestion with an appropriate restriction enzyme, preferably following amplification of the relevant DNA regions; S1 nuclease sequence analysis; non-denaturing
15 gel electrophoresis, preferably following amplification of the relevant DNA regions; conventional RFLP (restriction fragment length polymorphism) assays; selective DNA amplification using oligonucleotides which are matched for the wild-type sequence and unmatched for the mutant sequence or *vice versa*; or the selective introduction of a restriction
20 site using a PCR (or similar) primer matched for the wild-type or mutant genotype, followed by a restriction digest. The assay may be indirect, ie capable of detecting a mutation at another position or gene which is known to be linked to one or more of the mutant positions. The probes and primers may be fragments of DNA isolated from nature or may be
25 synthetic.

A non-denaturing gel may be used to detect differing lengths of fragments resulting from digestion with an appropriate restriction enzyme. The DNA is usually amplified before digestion, for example using the
30 polymerase chain reaction (PCR) method and modifications thereof.

Amplification of DNA may be achieved by the established PCR method as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491 or by developments thereof or alternatives such as the ligase chain reaction, QB replicase and nucleic acid sequence-based amplification.

5

An "appropriate restriction enzyme" is one which will recognise and cut the wild-type sequence and not the mutated sequence or *vice versa*. The sequence which is recognised and cut by the restriction enzyme (or not, as the case may be) can be present as a consequence of the mutation or it can be introduced into the normal or mutant allele using mismatched oligonucleotides in the PCR reaction. It is convenient if the enzyme cuts DNA only infrequently, in other words if it recognises a sequence which occurs only rarely.

15 In another method, a pair of PCR primers are used which match (ie hybridise to) either the wild-type genotype or the mutant genotype but not both. Whether amplified DNA is produced will then indicate the wild-type or mutant genotype (and hence phenotype). However, this method relies partly on a negative result (ie the absence of amplified DNA) which could be due to a technical failure. It is therefore less reliable and/or requires additional control experiments.

A preferable method employs similar PCR primers but, as well as hybridising to only one of the wild-type or mutant sequences, they introduce a restriction site which is not otherwise there in either the wild-type or mutant sequences.

In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme sites appended to their 5' ends. Thus, all nucleotides of the primers are derived from the gene sequence of interest

30

or sequences adjacent to that gene except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art. The primers themselves can be synthesized using techniques which are well known in the art. Generally, the primers can be made
5 using synthesizing machines which are commercially available.

A fourth aspect of the invention provides a kit of parts for determining a fetal abnormality comprising (a) means for determining whether a cell contains or expresses an adult liver component and (b) means for
10 analysing a cell for an abnormality.

The means for determining whether a cell contains or expresses an adult liver component includes the aforementioned binding moieties and reagents (eg substrates) for detecting the said enzymes when the adult liver
15 component is an enzyme and reagents such as PCR primers, deoxynucleotides and a DNA polymerase for detecting the said mRNA when the adult liver component is an mRNA. Antibodies, or fragments or derivatives thereof, to the adult liver components are preferred. It is particularly preferred if the adult liver component is a cell surface
20 component as described above.

The means for analysing a cell for an abnormality include any such means. It is particularly preferred if the means are means for detecting a genetic abnormality. Suitable such means therefore includes nucleic acid
25 molecules, such as PCR primers and probes, which selectively hybridise to a gene of interest, ie one in which a genetic abnormality is being sought.

A further aspect of the invention provides the use of a binding moiety
30 which binding moiety binds to an adult liver component in a method of

determining a fetal abnormality the method comprising identifying or isolating embryonic or fetal cells according to the method of the first or second aspect of the invention and analysing said embryonic or fetal cell for said fetal abnormality.

5

Preferably, the binding moiety is used in a method where the fetal cell abnormality is determined by analysing genetic material, for example for chromosomal abnormalities or mutations in DNA.

- 10 A still further aspect of the invention provides the use of a means for determining whether a cell contains or expresses an adult liver component for identifying or isolating an embryonic or fetal red blood cell.

The means for determining whether a cell contains or expresses an adult
15 liver component are those as described in the fourth aspect of the invention.

The invention will now be described in more detail with reference to the following Examples and Figure wherein:

20

Figure 1 (95chorion aGLUT2 1;100X40RH) shows a human chorionic villus at 56 post-ovulatory days showing intense alpha GLUT 2 immunoreactivity in a megaloblast (arrow) and no reactivity in a normocyte (arrowhead) within a fetal chorionic blood vessel (bv). The syncytiotrophoblastic (s) and cytotrophoblastic (c) layers are minimally
25 immunoreactive.

Example 1: Antibodies directed at adult liver components

- 30 Antibodies directed against purified rat liver testosterone/4-nitrophenol

UDPGT are raised in Suffolk Cross Blackface sheep by a combination of intradermal and subcutaneous injection. IgG is prepared from the antiserum by a combination of ammonium sulphate precipitation and diethyl aminoethyl-cellulose chromatography (Burchell *et al* (1984) *Biochem. Soc. Trans.* **12**, 50). Typically sheep antirat liver testosterone/4-nitrophenol UDPGT antibody preparation (RAL 1) inhibit UDPGT activity towards bilirubin, testosterone, 1-naphthol, androsterone, estrone, and morphine, and immunoblotting confirms a broad spectrum of cross-reactivity to multiple isoforms in rat and human adult and fetal liver microsomes.

Monospecific polyclonal antisera to the catalytic subunit of the microsomal glucose-6-phosphate system, T2, and T3 are each raised in Cheviot sheep by 3 subcutaneous injections of 80 μ g of purified protein and Freund's complete adjuvant as described (Burchell & Waddell: Genetic deficiencies of the hepatic microsomal glucose-6-phosphatase system, in Randle *et al* (eds): Genetics and Human Nutrition, London, UK, Libbey, 1990, p93; Waddell *et al* (1991) *Biochem. J.* **275**, 363; Burchell & Cain (1985) *Diabetologia* **28**, 852). Preimmune serum is obtained from each sheep before injection with antigen. The glucose-6-phosphatase enzyme, T2, and T3 used as antigens are all isolated from starved Wistar rat hepatic microsomes. Antisera are further purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and affinity purification using protein G columns. The antibody preparations, although raised against rat liver proteins, have been shown many times to each cross-react well with the respective human proteins as judged by immunoblot analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Antisera are raised in rabbits to methyl-moximated PGEM (PGEM-MOX) coupled to bovine serum albumin (Kelly *et al* (1986) *Prostaglandins*

Leukot. Med. 24, 1). In radioimmuno-assay, the PGEM-MOX antiserum has only a 0.05% cross-reactivity with PGE₂. Cross-reactions of PGEM-MOX antiserum to other methyl-moximated PGs are typically less than 0.02%, except for 15-keto-PGE₂ (12%) and 15-keto-PGE_{2α} (0.9%). The percentage cross-reactivities of PGEM-MOX antiserum to other PGs are typically less than 0.02%, except for 6,15-diketo-13,14-dihydro-PGE_{2α} (0.35%); 13,14-dihydro-PGE_{2α} (2%); and 15-keto-PGF_{2α} (4%). Cross-reactivity of Gemeprost to PGEM-MOX antiserum is typically less than 0.03% and to PGFM-MOX antiserum, 0.03%. Antibody specificity of PGEM-MOX antiserum for immunohistochemistry is shown by selective absorption of immunostaining by PGEM-MOX (Hume *et al* (1993) *Exp. Lung Res.* 19, 361). Anti-PGHS-1, a polyclonal goat IgG fraction with cross-reactivity to PGHS-1 from a number of mammalian species but negligible reactivity to PGHS-2, and a monoclonal 15-hydroxyprostaglandin dehydrogenase antibody are purchased from Oxford Biomedical Research, Inc (Oxford, MI).

PGE₂ is conjugated to keyhole limpet hemocyanin and injected intradermally into sheep. The resultant PGE₂-antiserum, on radioimmunoassay, is highly group-selective, with minimal cross-reactivity between the F and E series of PGs. Cross-reactivity of Gemeprost to PGE₂-antiserum is typically less than 1%. Antisera specificity for immunohistochemistry is tested by selectivity of absorption of immunostaining by PGE₂ in human fetal lung (Hume *et al* (1992) *Exp. Lung Res.* 18, 259).

Antibodies to purified cytochrome P450s are isolated as described in Wolf *et al* (1984) *Carcinogenesis* 5, 993. The isozyme specificities of the antisera made in this way have been shown by immunoblot analysis with expressed human recombinant cytochrome P450 proteins (Forrester *et al*

(1992) *Biochem. J.* **281**, 359). NADPH-cytochrome P450 oxidoreductase is purified (Wolf *et al* (1984) *Carcinogenesis* **5**, 993) and antibodies raised in rabbits are shown to cross-react with the human enzyme on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Smith *et al* (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8710).

In addition to antibodies made using isolated proteins as an antigen, antipeptide antibodies are also useful.

Antipeptide antibodies are synthesised on the basis of known sequence information and/or larger portions of proteins which have been generated using portions of the cDNA or gene linked to appropriate sequences which facilitate isolation after overexpression in a suitable system, eg bacteria. A series of antipeptide antibodies against portions of the human glucose-6-phosphatase enzyme (CSHIHSIYNASLKKY (SEQ ID No: 1); CMNVLHDFGIQSTHY (SEQ ID No: 2); CLAQVLGQPHKKSL (SEQ ID No: 3); and CLSRIYLAAHFPHQ (SEQ ID No: 4)) have been made as follows.

These peptides were conjugated to keyhole limpet hemocyanin and injected into sheep by intradermal and subcutaneous routes. The resultant antipeptide antiserum have been shown to cross react and can be used for immunohistochemical detection of glucose-6-phosphatase containing cells including fetal and embryonic cells.

Example 2: Combined immunocytochemical and fluorescence *in situ* hybridisation analysis of a maternal blood sample to detect trisomy 21

Blood samples and cell preparation. Peripheral venous blood samples (EDTA) are obtained from a pregnant female in the first trimester. Five ml aliquots of blood are carefully layered over 3.5 ml aliquots of

Polymorphoprep (Nycomed, Norway) in 15 ml tubes which are then spun at 500 g for 30 min at room temperature. Mononuclear cells at the plasma/Polymorphoprep interface (upper of the two bands obtained) are harvested using a Pasteur pipette and dispensed into a clean tube. The cells are washed three times using 5 ml of cold phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 5 mM ethylenediaminetetra-acetic acid (EDTA) followed by a 10 min spin at 400 g each time. The cell pellets are finally resuspended in PBS/BSA/EDTA at a concentration of 10^6 cells/ml.

10

Slide preparation. Aliquots (100 μ l) of blood mononuclear cells are cytocentrifuged onto glass slides (Cytospin, Shandon) and air dried overnight.

15 *Immunocytochemistry.* The following polyclonal and monoclonal antibodies are used in the immunocytochemical procedure: (a) a mouse monoclonal antibody directed to glucose-6-phosphatase enzyme (b) rabbit anti-mouse IgG (DAKO) at 1:25, and (c) mouse PAP (DAKO) at 1:100. All antibodies are diluted in TBS containing 20% normal rabbit serum (Serotec) and antibody incubations are carried out in a humidified chamber at room temperature.

Dry cytospins are fixed in acetone for 3 min and washed for 5 min in two changes of Tris-buffered saline (TBS). The standard PAP technique (Sternberger *et al*, 1970, *J. Histochem. Cytochem.* 18, 315-333) is modified. Briefly, endogenous peroxidase activity is blocked using 0.3% hydrogen peroxide in TBS for 30 min after which time slides are rinsed in two changes of TBS for 5 min. Cytospins are incubated with 20% normal rabbit serum in TBS for 5 min to block non-specific binding sites. After removal of most of the normal rabbit serum, cytospins are incubated

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with anti-G6Pase monoclonal antibody for 30 min. After washing the slides in TBS as before, followed by incubation with 20% normal rabbit serum in TBS for 5 min, cytopins are incubated with rabbit anti-mouse IgG for 30 min. The slides are washed as above and incubated with 20% normal rabbit serum in TBS for 5 min followed by mouse PAP for 30 min. The slides are then washed as above and two to three drops of 3,3-diaminobenzidine (DAB) substrate solution are added and incubated at room temperature for 7 min. The DAB solution is prepared by dissolving 2.5 mg of DAB (Sigma) in 5 ml of TBS followed by the addition 0.1 ml of freshly prepared 1% hydrogen peroxide immediately before use. The slides are washed in running tap water for 2 min followed by a 5 min incubation in copper sulphate solution (0.4 g copper sulphate, 0.72 g sodium chloride, 100 ml distilled water) and rinsed again in running tap water. In order to check the morphology of the cells after immunocytochemistry, the slides may be counterstained with Mayer's hematoxylin (Sigma) for 20 min, dehydrated through graded alcohols, and checked using a light microscope Zeiss Axioskop 20.

Immunohistochemistry on tissue sections is performed using anti-G6Pase monoclonal antibody and a standard PAP technique (Sternberger *et al*, 1970, *J. Histochem. Cytochem.* **18**, 315-333). Sections are lightly counterstained with haemotoxlyin, dehydrated through graded alcohol and cleared in xylene prior to coverslipping in synthetic resin.

Fluorescence in situ hybridisation (FISH). Following immunocytochemistry, cytopins are fixed in 3:1 (v/v) methanol:glacial acetic acid for 30 min at room temperature. The fixation is repeated with a fresh preparation of the same fixative for a further 30 min and with 70:30 (v/v) glacial acetic acid:water for 90 sec. The slides are washed for 5 min in two changes of PBS and then dehydrated through 70%, 85%, and

100% alcohol and air-dried at room temperature. FISH is performed using probes specific for human for human chromosome 21 directly labelled with fluorescein isothiocyanate (FITC). Briefly, hybridisation fluid is pipetted onto each cytospin and a coverslip coated with the fluorescent probes is placed on top and sealed with rubber solution. In the presence of hybridisation fluid, the probes are eluted from the coverslip and after denaturation of both probe and target DNA which occurred under the coverslip by heating the slides to 70°C, the probes are allowed to hybridise to the targets for 25 min at 37°C. After several post hybridisation washes the slides are mounted in antifade solution containing diamidino-2-phenyl-indole dihydrochloride (DAPI).

Microscopy. Slides are analysed using a Zeiss Axioskop 20 microscope equipped with a microscope illuminator with mercury vapour short-arc lamp HBO 50W and appropriate Zeiss filter combinations (02 for DAPI; 09 for FITC). The immunopositive cells are located using the microscope in the visible light mode, after which the visible light source is blocked and the microscope switched to fluorescence mode and slides analysed using each filter set. Cells are photographed on Fujichrome Provia 400 colour film (Fuji Photo Film Co., Tokyo, Japan) both in visible light mode and fluorescence mode. Black and white negatives and prints are then made from the colour film.

Results

25

Three positive fluorescent spots from chromosome 21 are present in embryonic or fetal cells derived from an affected fetus. In contrast, normal maternal cells and normal fetal cells give two positive fluorescent spots for chromosome 21. Fetal and maternal cells are distinguished by immunoreactivity to glucose-6-phosphatase, ie fetal red blood cells are

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immunopositive, maternal red blood cells immunonegative.

Example 3: Isolation of fetal cells and PCR analysis for Sickle cell anaemia and thalassaemia

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Figure 1 (95chorion aGLUT2 1;100X40RH) shows a human chorionic villus at 56 post-ovulatory days showing intense alpha GLUT 2 immunoreactivity in a megaloblast (arrow) and no reactivity in a normocyte (arrowhead) within a fetal chorionic blood vessel (bv). The syncytiotrophoblastic (s) and cytotrophoblastic (c) layers are minimally immunoreactive.

10

Fetal cells from maternal blood taken in the first trimester are isolated in the following way, and a PCR analysis for Sickle cell anaemia and thalassaemia undertaken.

15

Blood sample and cell separation. Peripheral blood (16-18 ml) from pregnant women in the first trimester is collected into EDTA Vacutainer tubes (Becton Dickinson, Rutherford, NJ). The blood is then diluted 1:2 with phosphate buffered saline (PBS), with each 15 ml layered over 10 ml Ficoll-paque plus (density 1.077 g/ml, Pharmacia Biotech, Piscatawa, NJ) and centrifuged at 400 g for 30 min at room temperature. The interphase cells are then carefully removed, washed twice with PBS supplemented with 5 mM EDTA and 0.5% bovine serum albumin (BSA), and resuspended in 80 μ l PBS/EDTA/BSA per each 10^7 cells.

20

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Magnetic activated cell sorting. Nucleated red cells were enriched by MiniMACS (Miltenyi Biotech, Inc., Sunnyvale, CA) following the manufacturer's protocol. An anti-GLUT2 antibody conjugated MACS Microbeads is added to the resuspended cells at the ratio of 20 μ l per each

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10⁷ cells. The mixture is then incubated for 15 min at 6-12°C in a refrigerator. The magnetic-bead labelled cell suspension is pipetted on top of a MiniMACS column and the unlabelled cells are then collected by pushing them out of the column using 1 ml buffer and a plunger.

5

The cells isolated in this way are substantially all early fetal or embryonic nucleated red blood cells.

PCR is performed as described (Saiki *et al* (1988) *Science* **239**, 487-491) in 50 µl reaction volume using a Perkin-Elmer DNA Thermal Cycler. Each amplification cycle consists of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, with 10 min final extension at 72°C in the last cycle. DNA from the fetal or embryonic cells eluted from the MiniMACS column are first amplified for 40 cycles and a fifth of the products is examined on an 8% acrylamide gel. If no specific or very weak PCR products are seen, an aliquot of 10 µl of the first round PCR products is amplified for another 20-30 cycles using the same conditions. The primers used to amplify the β-globin sequences are biotinated 'pco3' (5'-Biotin-ACACAAGTGTGTTCACTAGC-3' (SEQ ID No: 5)), biotinated 'β110' (5'-Biotin-AAAATAGACCAATAGGCAGA-3' (SEQ ID No: 6)) and biotinated 'China 2' (5'-Biotin-TGCAGCTTGTCACAGTGCAGCTCACT-3' (SEQ ID No: 7)). The 248-bp DNA amplified by 'pco3' and 'β110' is used for sickle gene detection. The 460-bp DNA amplified by 'pco3' and 'China 2' is used for β-thalassemia detection.

Reverse dot blot hybridization. Membrane strips containing multiple dots of immobilized normal and mutant oligonucleotide probes of β-globin sequences are prepared as described (Maggio *et al* (1993) *Blood* **81**, 239-242; Cai *et al* (1994) *Human Mutation* **3**, 59-63). The sequences of these

oligonucleotide probes for detecting mutations are as follows: for detecting the sickle gene, the normal probe is 5'-TGACTCCTGAGGAGAAGT-3' (SEQ ID No: 8) and the mutant probe is 5'-CAGACTTCTCCACAGGA-3' (SEQ ID No: 9); for detecting the
5 β 39 mutation, the normal probe is 5'-CTTGGACCCAGAGGTTCTT-3' (SEQ ID No: 10) and the mutant probe is 5'-AGAACCTCTAGGTCCAAGG-3' (SEQ ID No: 11); and for detecting the β 110 mutation, the normal probe is 5'-GAAAATAGACCAATAGGCAGA-3' (SEQ ID No: 12), and the mutant
10 probe is 5'-CTGCCTATTAGTCTATTTTC-3' (SEQ ID No: 13). The PCR products of the fetal cells are added to a 0.8 ml solution containing 2 x SSC/0.1% SDS. Strips containing the oligonucleotide probes are added and DNAs are denatured by boiling in a water bath for 5 min. Hybridization is carried out in a 42°C water bath overnight. The strips
15 are then washed in 0.5 x SSC/0.1% SDS at 42°C for 10 min, conjugated with streptavidin-HRP at room temperature for 15 min, and the colour developed with chromogenic substrate containing tetramethyl benzidine solution, Na citrate and H₂O₂ at room temperature until colours were visible whether or not the fetal cells have a sickle cell or thalassaemia
20 mutation can be detected by this method.

CLAIMS

1. A method of identifying embryonic or fetal red blood cells in a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising determining which cell or cells contain or express an adult liver component.
5
2. A method of isolating embryonic or fetal red blood cells from a sample containing maternal blood cells and embryonic or fetal red blood cells or both, the method comprising isolating the cells which contain or express an adult liver component.
10
3. A method according to Claim 1 or 2 wherein the said sample is a sample of blood from a pregnant female.
15
4. A method according to Claim 3 wherein the pregnant female is a human female and the sample is taken in the first trimester.
5. A method according to Claim 1 or 2 wherein the embryonic or fetal red blood cell is of the nucleated megaloblastic series.
20
6. A method according to any one of Claims 1 to 5 wherein the component is a protein.
- 25 7. A method according to any one of Claims 1 to 6 wherein the component is substantially absent from maternal cells of the maternal blood.
8. A method according to any one of Claim 1 or 2 wherein the protein is any one of a microsomal glucose-6-phosphatase enzyme, another
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protein component of the glucose-6-phosphatase system including the phosphate or glucose or glucose-6-phosphate transporters, a uridine diphosphate-glucuronosyltransferase, a cytochrome P450 isozyme, nicotinamide adenine dinucleotide phosphate cytochrome P450 reductase, glucose transporter 2, a P-glycoprotein, a MDRP, a MRP, γ -glutamyl transpeptidase, a lipoprotein receptor, an alkaline phosphatase, a bile salt transporter, a bile acid transporter, a hormone receptor, a multiple organic ion transporter, a bilirubin transporter or a bilirubin conjugate transporter.

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9. A method according to any one of the preceding claims wherein the adult liver component is a cell surface exposed component.

10. A method according to Claim 9 wherein the cell surface exposed component is an adult liver plasma membrane protein.

15

11. A method according to Claim 10 wherein the adult liver plasma membrane protein is any of glucose transporter 2, a P-glycoprotein, a MDRP, a MRP, γ -glutamyl transpeptidase, a lipoprotein receptor, an alkaline phosphatase, a bile salt transporter, a bile acid transporter, a hormone receptor, a multiple organic ion transporter, a bilirubin transporter or a bilirubin conjugate transporter.

20

12. A method according to any one of the preceding claims wherein the said sample is contacted with a binding moiety which moiety binds to said adult liver component and said embryonic or fetal cell is identified in or isolated from the sample by virtue of being bound to the binding moiety.

25

13. A method according to Claim 1 to 11 wherein said sample is

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contacted with a substrate for an enzyme, said enzyme being an adult liver component, and said embryonic or fetal cell is identified in or isolated from the sample by virtue of the product formed by action of said enzyme on said substrate.

5

14. A method according to Claim 12 wherein said binding moiety is an antibody or fragment or derivative thereof.

15. A method of isolating embryonic or fetal red blood cells from a sample according to Claim 12 or 14 wherein said binding moiety is immobilised to a solid support.

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16. A method according to Claim 12 or 14 wherein said binding moiety is detectably labelled or is capable of detection.

15

17. A method of isolating embryonic or fetal red blood cells from a sample according to Claim 16 wherein said label facilitates isolation of the cells.

18. A method according to Claim 13 wherein said product is fluorescent or coloured.

20

19. A method of determining a fetal abnormality the method comprising identifying or isolating embryonic or fetal cells according to the method of any one of the preceding claims and analysing said embryonic or fetal cell for said fetal abnormality.

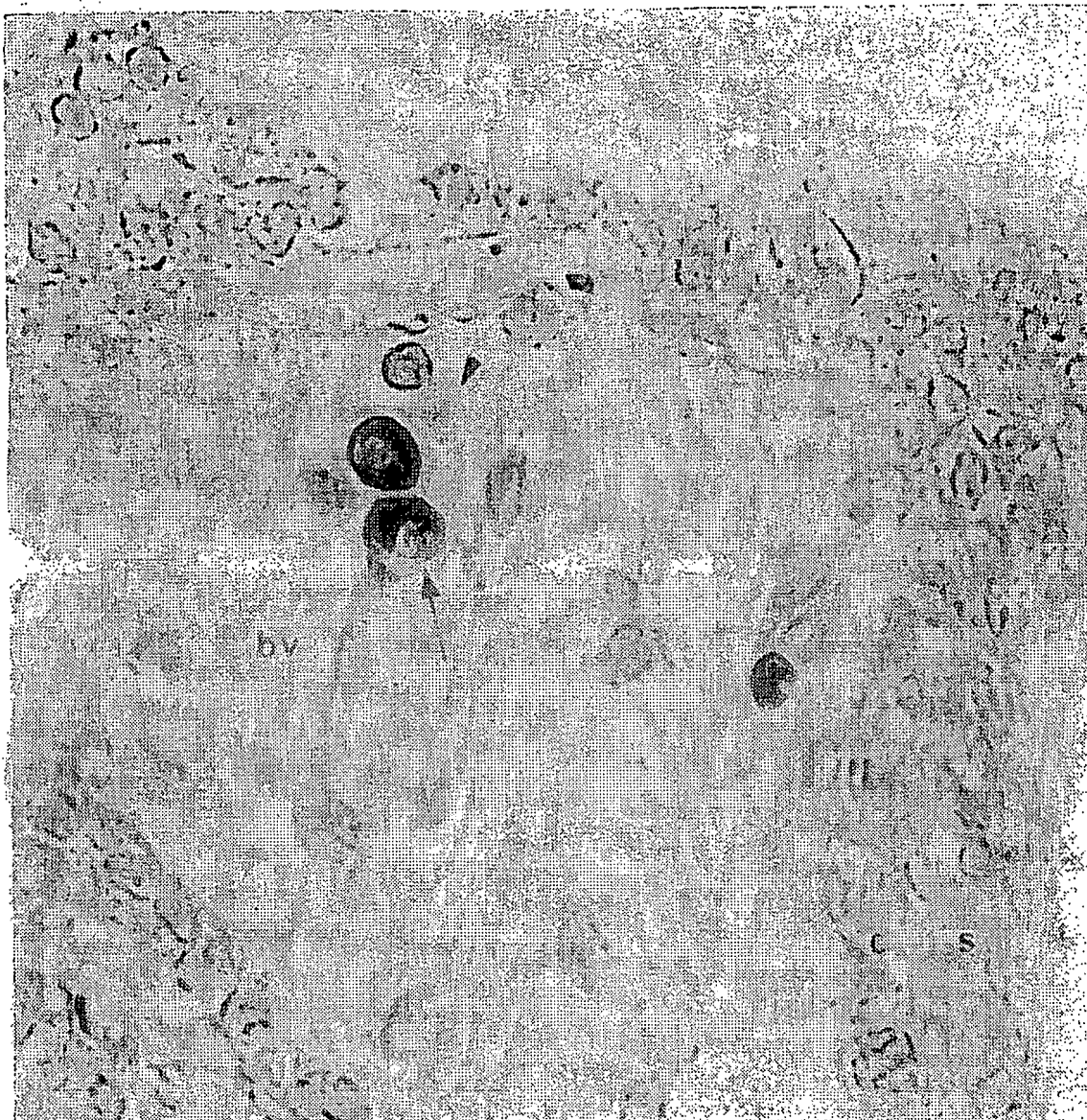
25

20. A method according to Claim 19 wherein the fetal cell abnormality is determined by analysing genetic material.

30

21. A method according to Claim 20 wherein chromosomal abnormalities are detected.
22. A method according to Claim 20 wherein mutations in the DNA
5 are detected.
23. A kit of parts for determining a fetal abnormality comprising (a) means for determining whether a cell contains or expresses an adult liver component and (b) means for analysing a cell for an
10 abnormality.
24. Use of a binding moiety as defined in Claim 12 in a method according to any one of Claims 19 to 22.
- 15 25. Use of a means for determining whether a cell contains or expresses an adult liver component for identifying or isolating an embryonic or fetal red blood cell.
26. Any novel method of determining a fetal abnormality by analysing
20 embryonic or fetal cells in maternal blood as herein described.
27. Any novel method of isolating or identifying an embryonic or fetal cell as herein described.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00656

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/573 G01N33/68 G01N33/569 G01N33/80

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 07660 A (CHILDRENS MEDICAL CENTER) 30 May 1991 see example 1	1-7,9, 12-25
Y	--- HUME R ET AL: "The ontogeny of the glucose -6-phosphatase enzyme in human embryonic and fetal red blood cells." EARLY HUMAN DEVELOPMENT 42 (2). 1995. 85-95. ISSN: 0378-3782, XP002065533 cited in the application see abstract	8
Y	--- MULIVOR R A ET AL: "PRE NATAL DIAGNOSIS OF HYPO PHOSPHATASIA GENETIC BIOCHEMICAL AND CLINICAL STUDIES." AM J HUM GENET 30 (3). 1978 271-282. CODEN: AJHGAG ISSN: 0002-9297, XP002065534 see table 4 --- -/--	10,11



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Interr 1a/ Application No

PCT/GB 98/00656

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUME R ET AL: "The ontogeny of key endoplasmic reticulum proteins in human embryonic and fetal red blood cells." BLOOD 87 (2). 1996. 762-770. ISSN: 0006-4971, XP002065535 cited in the application see page 762 - page 763 ---	8
Y	WO 96 09409 A (MILTENYI BIOTECH INC) 28 March 1996 see page 13 - page 20 ---	9-25
Y	WO 91 14768 A (GENETYPE AG) 3 October 1991 see examples 1-9 ---	9-25
A	SHIN Y S ET AL: "PRE NATAL DIAGNOSIS OF GALACTOSEMIA AND PROPERTIES OF GALACTOSE 1 PHOSPHATE URIDYL TRANSFERASE EC-2.7.7.12 IN ERYTHROCYTES OF GALACTOSEMIC VARIANTS AS WELL AS IN HUMAN FETAL AND ADULT ORGANS." CLIN CHIM ACTA 128 (2-3). 1983. 271-282. CODEN: CCATAR ISSN: 0009-8981, XP002065536 see page 274 ---	1,2
A	MOGHRABI N ET AL: "Cosegregation of intragenic markers with a novel mutation that causes Crigler-Najjar syndrome type I: Implication in carrier detection and prenatal diagnosis." AMERICAN JOURNAL OF HUMAN GENETICS 53 (3). 1993. 722-729. ISSN: 0002-9297, XP002065537 see abstract -----	1,7

INTERNATIONAL SEARCH REPORT

Ir. ational application No.

PCT/GB 98/00656

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
CLAIMS 26 AND 27 RELY, CONTRARY TO RULE 6.2A PCT, ON REFERENCES TO THE DESCRIPTION, AND ARE CONSEQUENTLY OF NEBULOUS MEANING.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/00656

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9609409 A	28-03-1996	AU 3593595 A CA 2200294 A EP 0778899 A	09-04-1996 28-03-1996 18-06-1997
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